# THE FIBRILLAR STRUCTURE OF COLLAGEN FROM ENZYME- AND LIME-UNHAIRED HIDES*


## ABSTRACT

Steerhides unhaired both by liming and enzymatic action have been investigated by X-ray and electron microscopic techniques. No major change, deterioration, or breakup of the collagen fibrils was observed. However, there is good evidence, from the shift of X-ray diffraction peaks, that treatment by a proteolytic enzyme produces shrinkage of all molecular dimensions by 5–10%. Subsequent liming of the hide restores the dimensions to approximately the original values. Two types of collagen structure have been observed in the electron microscope: Type W, characterized by contrasting wide bands with the usual 640 A spacing, and Type N, characterized by contrasting narrow bands or subperiods. The liming process has the effect of sharpening and increasing the contrast of the fine structure of both types of collagen. The enzyme treatments have the greatest modifying effect on the fibril fine structure: the appearance of longitudinal striations and the suppression of contrast between the bands of Type W. The modifications are interpreted in terms of the attack of enzymes on the end regions of the tropocollagen molecule where the basic groups are found which normally bind the PTA used to stain electron microscope specimens.


## INTRODUCTION

It is possible to unhair hides either by liming or by enzymatic action. However, the leathers which are produced subsequent to the depilation are quite different in their physical properties. Leather from a limed hide is softer and more pliable and shows less shrinkage than hides which have been

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TABLE I

PERIODIC SPACINGS OF PROCESSED STEERHIDES

| Peak | Shaved | Lime | Enzyme A | Enzyme A 1-Day Lime | Enzyme A 3-Day Lime | Enzyme B | Enzyme B 1-Day Lime | Enzyme B 3-Day Lime |
|---|---|---|---|---|---|---|---|---|
| *a* | 11.9A | 11.3A | 11.3A | 12.4A | 12.4A | 11.9A | 12.2A | 11.8A |
| *b* | 7.58 | 7.36 | 6.77 | 7.36 | 7.37 | 7.58 | 7.70 | 7.44 |
| *c* | 4.80 | 4.75 | 4.60 | 5.06 | 5.00 | 4.85 | 5.02 | 4.85 |
| *d* | 2.98 | 2.92 | 2.88 | 3.09 | 3.09 | 2.98 | 3.02 | 2.98 |

For gross structure studies, several drops of the aqueous suspension were sprayed onto an electron microscope specimen grid which had been precoated with parlodian. The grids were then air-dried, shadowed with metal in a vacuum evaporator (14° shadowing angle), and finally given a thin deposition of carbon to provide strength to the parlodian substrate. Thermal degradation of the collagen during the shadowing was prevented by the use of radiation shields. An 80% platinum–20% palladium alloy was employed for shadowing.

Study of the interband structure of collagen requires preferential staining of the fibers. Four drops of 1% phosphotungstic acid (PTA) solution were added to 10 cc. of the aqueous hide dispersion, and the mixture was allowed to stand for 48 hr. in the cold. A few drops of the mixture were then sprayed on a carbon grid and examined at high magnification in an Hitachi HU-11 electron microscope.

## RESULTS AND DISCUSSION

**X-ray studies.**—The microdensitometer trace of a typical X-ray diffraction photograph obtained from a dry hide sample is reproduced in Fig. 1. This pattern agrees very well with the standard pattern of $\gamma$- proteins (2). The peaks marked *a*, *b*, *c*, and *d* are generally interpreted in the following way:

Peak *a*—corresponds to the approximately 11 A spacing between adjacent tropocollagen (TC) molecules which make up the normal collagen fibril.

Peak *b*—corresponds to an approximate 7.5 A spacing characteristic of the side chain bond length in TC.

Peak *c*—a diffuse line corresponding to the 4.4 A spacing between individual polypeptide chains in the TC molecule.

Peak *d*—corresponds to the 2.86 A spacing between residues along the polypeptide chains of TC.

In the diffraction photograph each peak was recorded as a continuous ring of uniform density. Thus, one can say the fibers in the specimen responsible

unhaired by enzyme treatment. The possibility exists that the two unhairing processes affect differently the basic collagen constituents of the hide. A companion paper (1) (hereafter referred to as Paper I) describes the results of a chemical study undertaken to reveal the nature of the attack on the collagen by the process reagents. The present paper describes a physical investigation of the hide collagen carried out with X-ray and electron microscope techniques.

## EXPERIMENTAL METHODS

**Preparation of hides.**—Hide samples were given the following unhairing treatment:

Liming
Enzyme A—H.T. Proteolytic (Miles Laboratories, Inc.*)
Enzyme A + 3 days lime
Enzyme B—M-Zyme (Merck)
Enzyme B + 3 days lime
Shaving with a razor

The detailed procedures by which these hides were then prepared for physical observation have been given in Paper I and will not be repeated here.

**X-ray studies.**—The differently treated hide samples were cut into specimens about 1 mm. thick for X-ray analysis. These specimens were then placed at the exit slit of a pinhole collimator (1.0 mm. slits), and the transmission Laue pattern was recorded with a flat film camera. The specimen-film distance was 40 mm., and typical exposure times were 3–4 hr. All specimens were examined in the dry condition, with no special precautions to control the humidity of the air surrounding the samples. (The exposures were made during early winter when the room humidity is normally 25–30%.)

To measure the periodic spacings responsible for the diffraction pattern, the photographs were converted to line profiles by means of a microdensitometer. Each spacing reported here (Table I) is the average of at least three patterns recorded from the same hide sample. The standard deviation, based on the scatter of observed spacings, was 2%.

**Electron microscope studies.**—For these studies it was first necessary to form dispersions of sufficient concentration to allow electron microscopic examination of the hides. These dispersions were formed by thoroughly soaking pieces of the hide in water and then dispersing them by mechanical agitation in a blender. Concentrations obtained in this manner were estimated to be of the order of 0.5% collagen by weight. This is adequate for use in the electron microscope.

*Mentioning brand or firm names does not constitute an endorsement of the Department of Agriculture over others of a similar nature not mentioned.

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In the diffraction photograph each peak was recorded as a continuous ring of uniform density. Thus, one can say the fibers in the specimen responsible

for the diffraction are randomly oriented. The average periodic spacing for collagen calculated from diffraction patterns obtained from the variously treated hides is given in Table I. Differences of less than 2% between these hides should be ignored since they are comparable to the experimental error.

For an analysis of the different process treatments, the periodic spacings found for the shaved hide are taken as the norm. The following paragraphs discuss particular X-ray results.

*Lime.*—The intermolecular spacing (corresponding to peak *a*) within the collagen fibril is the only dimension to change significantly following the lime treatment. The over-all results of the observed decrease would be to shrink the average diameter of the collagen fibril by about 5 %.

*Enzyme A.*—Treatment with Enzyme A causes each recorded spacing to decrease with respect to the shaved hide. This over-all shrinkage of the molcular dimension is consistent with the macroscopic observation that enzyme-treated hides as prepared in this study (including acetone dehydration) shrink and lose their pliability. One-day liming after treatment with Enzyme A reverses the effect so that all but one of the spacings becomes larger than those of either the shaved or the limed hide. Increasing the liming period three days does not significantly alter the pattern obtained in Enzyme A and one-day liming.

*Enzyme B.*—The X-ray diffraction patterns show no significant difference between the hide unhaired with Enzyme B and the raw shaved hide. One-day liming after unhairing with Enzyme B produces a small increase in the spacings. After three-day liming the hide is restored to the same condition as found for a hide treated with Enzyme B only. Because of the small differences involved and the difficulty in measuring the exact position of the X-ray diffraction peak, it is believed that the variation observed after liming is more statistical than real.

**Electron microscope studies.**—Two methods were used for the observation of collagen fibrils in the electron microscope. A quick survey of gross structure was made using samples shadowed with a platinum-paladium alloy. The detailed interband structure was studied by staining the various collagen samples with PTA. Photographs of the shadowed specimens showed no gross differences between samples. The 640 A period characteristic of collagen was clearly visible in all pictures, but variation in the magnitude of this spacing from sample to sample was no more than that normally found among fibrils in the same sample.

The study of the stained collagen samples has revealed the existence of two major types of collagen found in all of the samples. These types have been designated Type W and Type N and their characteristics are listed below.

*Type W*: Smooth cylindrical shape with highly contrasting *wide* bands.

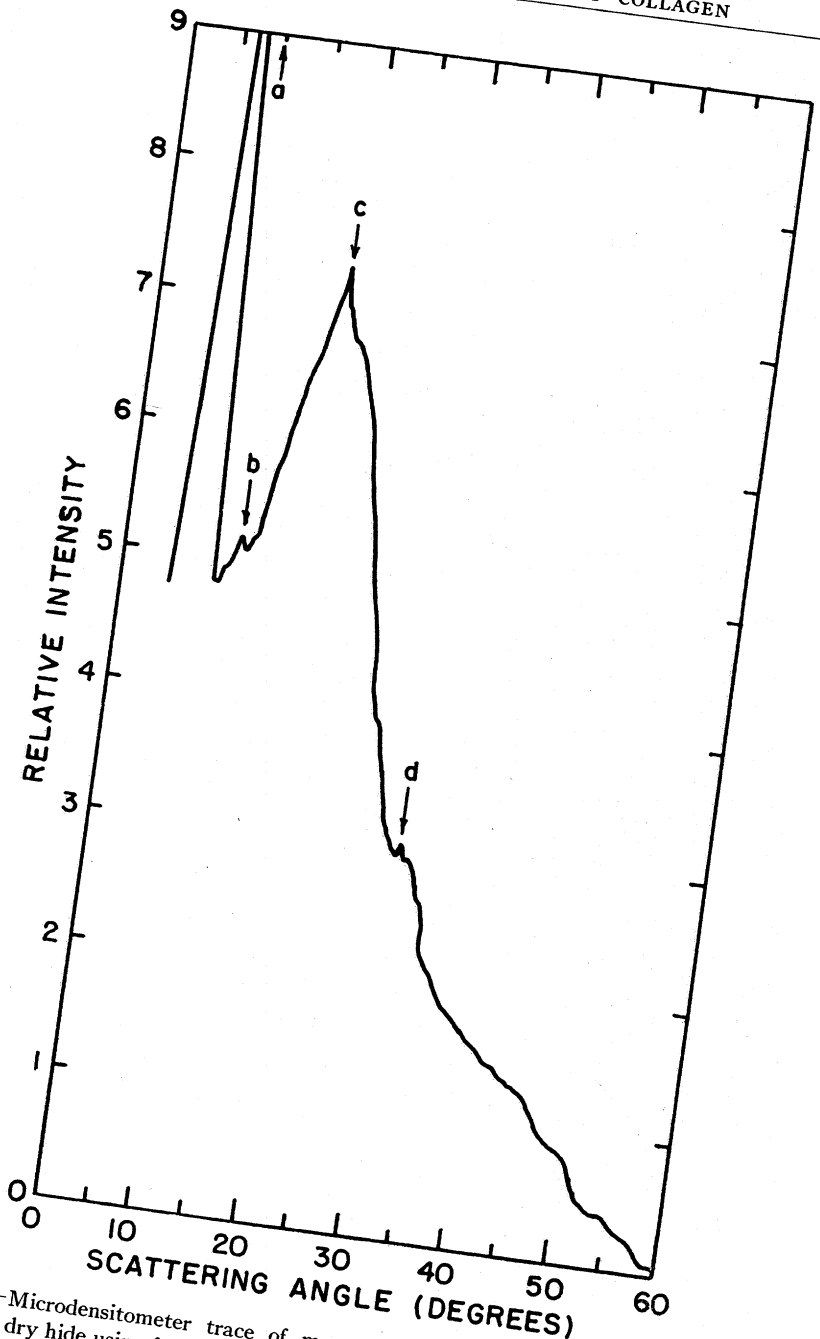


FIGURE 1.—Microdensitometer trace of monochromatic-pinhole X-ray pattern from dry hide using forward-reflection camera and Cu $K_\alpha$ Radiation ($\lambda$—1.54Å)

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*Type W*: Smooth cylindrical shape with highly contrasting *wide* bands.

Interpretation of the stained collagen micrographs must be made in terms of the results of Kühn (3) who found that PTA is preferentially absorbed on basic, chiefly arginine, residues. The rather large phosphotungstate ion is bound by the mutual cooperation of several polar side chains on adjacent TC molecules. These basic groups are found in higher concentration at the ends of the TC molecule, thus giving rise to the usual band contrast in stained collagen micrographs.

*Lime.*—It should first be stated that both Type W and Type N collagen observed in limed hides have considerably sharper structure and enhanced contrast over the shaved specimens. Because of the nature of PTA absorption, it can be concluded that the liming process probably causes an increase in the availability of basic arginine groups at certain preferred intervals along the TC chain. Furthermore, it is probable that there has been some absorption of calcium during the liming process, and this element will also act as a stain. The influence of the lime might also be due, in part, to the removal of globulins and albumin from the hide. In addition to the normal Type W and Type N collagen, one also observes frequently a modified Type W in which narrow longitudinal striations have developed in the dark bands (Fig. 3). This change is indicative of some rearrangement of sites at which PTA is absorbed.

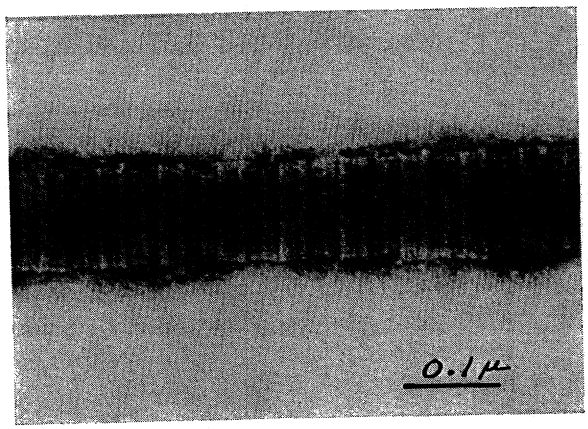
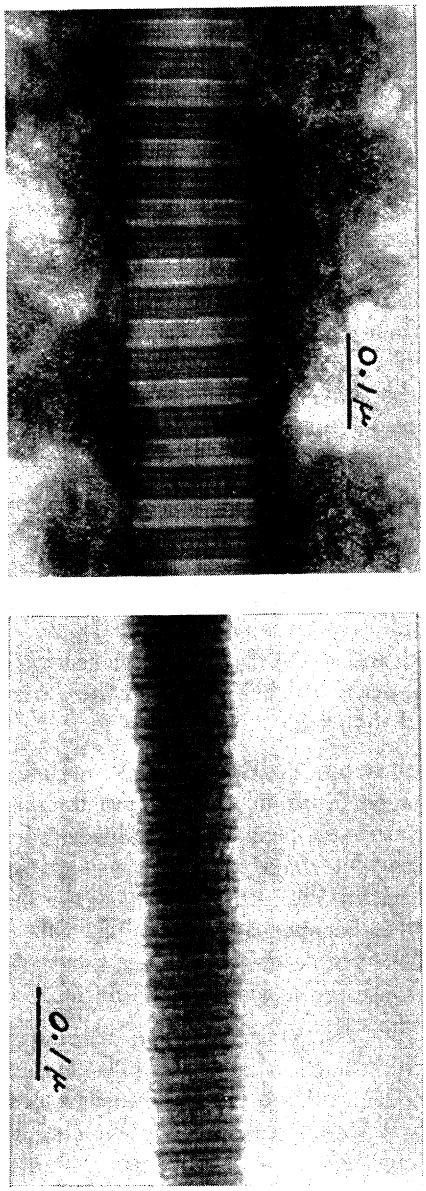



FIGURE 3.—Collagen fibrils from lime-unhaired hides—Type W with longitudinal striations.

*Enzymes A and B.*—In addition to the presence of the usual Type W and Type N collagen one finds that treatment with either enzyme results in a major modification of Type W as shown in Fig. 4. In addition to the longitudinal striations which were introduced by the liming process, the contrast is lost between the wide transverse bands. Type N also suffers a general loss in sharpness (Fig. 4B). In addition to these modifications, the hide treated with

*Type N*: Corrugated cylindrical shapes with contrasting *narrow* bands (subperiods).

Good examples of Type W and Type N are shown in Figs. 2A and 2B respectively. In addition to these two major types, characteristic modifications of Type W were found following the various process treatments.




A B

FIGURE 2.—Collagen fibrils from lime-unhaired hides. (*a*) Type W; (*b*) Type N.

All these observations are attributable to a general change in the internal structure and periodicity normally associated with native collagen. Apparently the basic groups responsible for PTA absorption become either randomized in position (loss in sharpness) or modified so that they no longer accept the stain (loss in contrast, Type W).

The results of Schmitt and Hodge (4) are helpful in this interpretation. These workers found that proteolytic enzymes such as trypsin and pepsin attacked the end regions of TC and impaired the end-to-end polymerization properties of the molecules which are responsible for the buildup of the usual collagen fibril. Since these end regions are also the places at which PTA is most likely to be bound, our results indicate that the treatments with Enzymes A and B resulted in a mild attack on the end groups of TC sufficient to destroy a portion of the normal periodic fibril structure. Rupture of some of the end-to-end TC linkages would tend to randomize the location of PTA absorption, and chemical changes in the end groups (Paper I) would suppress PTA absorption.

Liming following the enzyme treatment does not produce much change in Type W or its modifications. However, Type N collagen does regain some of its original sharpness and closely resembles the Type N collagen found in limed hides (Fig. 2B). Three-day treatment with lime following Enzyme B unhairing restored a great deal of the original sharpness of band structure. These results indicate that the end-to-end bonding of TC is somewhat restored by the lime treatment.

## CONCLUSIONS

Important conclusions to be drawn from this X-ray and electron microscope study of differently treated hides can be listed as follows:

1. The different treatments produced no major change, deterioration, or breakup of the collagen fibrils.

2. There is good evidence that treatment by Enzyme A produces shrinkage of all molecular dimensions by 5–10%. Subsequent liming of the hide restored these dimensions to approximately their original values.

3. Two types of collagen structures have been observed and designated as Type W and Type N. The liming process has the effect of sharpening and increasing the contrast of the fine structure of both these types of collagen. This result is most probably due to an increase in the availability of groups which are efficient in binding the PTA stain, and to the fact that calcium itself may act as a stain.

4. The two enzyme treatments have the greatest modifying effect on the fibril fine structure, characterized by the appearance of longitudinal striations, the suppression of contrast between the bands, and a general loss in sharpness

of band structure. These modifications can be interpreted in terms of a mild enzyme attack on the end regions of TC. A portion of the end-to-end linkages is apparently ruptured, and some chemical modification of the basic groups (arginine) occurs. Subsequent liming evidently restores some of the ruptured linkages.

## ACKNOWLEDGMENTS

It is a pleasure to acknowledge the expert help of Mr. Gordon Shaw who prepared the stained collagen specimens and carried out the electron microscopy. Dr. Robert Hight obtained the X-ray diffraction patterns. Numerous discussions with Mr. C. J. W. Wiegand are gratefully acknowledged.

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